PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

4153

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/55, 9/20, C11D 3/386	A1	(11) International Publication Number: WO 95/22615
		(43) International Publication Date: 24 August 1995 (24.08.95)

(21) International Application Number: PCT/DK95/00079

(22) International Filing Date: 22 February 1995 (22.02.95)

(30) Priority Data:
0217/94 22 February 1994 (22.02.94) DK

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD OF PREPARING A VARIANT OF A LIPOLYTIC ENZYME

(57) Abstract

A method of preparing a variant of a parent lipolytic enzyme, which method comprises: (a) subjecting a DNA sequence encoding the parent lipolytic enzyme to random mutagenesis, (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and (c) screening for host cells expressing a mutated lipolytic enzyme which has a decreased dependance to calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme.

A METHOD OF PREPARING A VARIANT OF A LIPOLYTIC ENZYME

FIELD OF THE INVENTION

The present invention relates to a method of preparing a variant of a parent lipolytic enzyme and to variants prepared by the method. Furthermore, the invention relates to a DNA construct encoding a variant of the invention, an expression vector and host cell comprising the DNA construct and a detergent additive or a detergent composition comprising a variant.

BACKGROUND OF THE INVENTION

For a number of years lipolytic enzymes have been used as detergent enzymes, i.e. to remove lipid or fatty stains from clothes and other textiles.

For instance, various microbial lipases have been suggested as detergent enzymes. Examples of such lipases include a *Humicola lanuginosa* lipase, e.g. described in EP 258 068 and EP 305 216, a *Rhizomucor miehei* lipase, e.g. as described in EP 238 023, a *Candida* lipase, such as a *C. antarctica* lipase, e.g. the *C. antarctica* lipase A or B described in EP 214 761, a *Pseudomonas* lipase such as a *P. alcaligenes* and *P. pseudoalcaligenes* lipase, e.g. as described in EP 218 272, a *P. cepacia* lipase, e.g. as described in EP 331 376, a *Bacillus* lipase, e.g. a *B. subtilis* lipase (Dartois et al., 1993), a *B. stearothermophilus* lipase (JP 64/744992) and a *B. pumilus* lipase (EP 91 00664).

Furthermore, a number of cloned lipases have been described, including the *Penicillium camembertii* lipase described by Yamaguchi, S. et al., 1991, the *Geotricum candidum* lipase (Schimada, Y. et al., 1989), and various *Rhizopus* lipases such as a *R. delemar* lipase (Hass, M.J et al., 1991), a *R. niveus* lipase (Kugimiya, W. 1992), and a *R. oryzae* lipase.

A need exists for novel lipolytic enzymes having improved washing and/or dishwashing properties, and the object of the present invention is to prepare such enzymes.

BRIEF DISCLOSURE OF THE INVENTION

The present inventors have now developed a novel method of preparing variants of lipolytic enzymes having improved washing and/or dishwashing performance as compared to their parent enzymes. The method is based on random or localized random mutagenesis of DNA sequences encoding a lipolytic enzyme.

More specifically, in a first aspect the invention relates to a method of preparing a variant of a parent lipolytic enzyme, which method comprises

(a) subjecting a DNA sequence encoding the parent lipolytic enzyme to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a mutated lipolytic enzyme which has a decreased dependance to calcium and/or an improved tolerance towards a detergent or one or more detergent components as compared to the parent lipolytic enzyme.

In the present context, the term "lipolytic enzyme" is intended to indicate an enzyme exhibiting a lipid degrading capability, such as a capability of degrading a triglycerid or a phospholipid. The lipolytic enzyme may, e.g., be a lipase, a phospholipase, an esterase or a cutinase.

The term "random mutagenesis" is intended to be understood in a conventional manner, i.e. to indicate an introduction of one or more mutations at random positions of the parent enzyme (i.e. as opposed to site-specific mutagenesis). The random mutations are typically introduced by exposing a large number

The screening criteria defined in step c) of the method of the invention may be determined by any suitable methods known in the art. A particular suitable assay developed for the present purpose is described in the Materials and Methods section below.

In a further aspect the invention relates to a DNA construct comprising a mutated DNA sequence encoding a variant of a lipolytic enzyme which has a decreased dependance to calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme, which DNA sequence is isolated from the host cell selected in step (c) of the method of the invention.

In a still further aspect the invention relates to a recombinant expression vector carrying the DNA construct, a cell which is transformed with the DNA construct or the vector as well as a method of producing the variant of the parent lipolytic enzyme by culturing said cell under conditions conducive to the production of the variant, after which the variant is recovered from the culture.

In final aspects the invention relates to a variant of a lipolytic enzyme and the use of said variant as a detergent enzyme, in particular for washing or dishwashing, and to a detergent additive and a detergent composition comprising the variant.

DETAILED DISCLOSURE OF THE INVENTION

Cloning a DNA sequence encoding a parent lipolytic enzyme

The DNA sequence encoding a parent lipolytic enzyme to be subjected to random mutagenesis in accordance with the present invention may be isolated from any cell or microorganism producing the parent enzyme in question by use of methods known in the art.

Random mutagenesis

The random mutagenesis of the DNA sequence encoding the parent lipolytic enzyme to be performed in accordance with step a) of the method of the invention may conveniently be performed by use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents.

The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose includes ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions wanted to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the lipolytic enzyme by any

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are given below. The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

It will be understood that the screening criteria mentioned in step (c) above have been carefully selected. Thus, without being limited to any theory the screening for a decreased dependency to calcium is believed to result in variants having an over-all improved performance in that the requirement for calcium may be considered a limiting factor for optimal activity, in particular under conditions where only low amounts of free calcium ions are present. In connection with detergent lipases the free calcium ions required are normally provided from the washing water and thus, the lipolytic activity is dependent on the calcium content of the water.

The detergent or detergent component towards which the variant has improved tolerance may be of any type, e.g. as further described below. Preferably, the detergent component is a non-ionic, anionic, kationic, zwitterionic or amphoteric surfactant. Examples of non-ionic surfactants include an alcohol ethoxylate, examples of anionic surfactants include LAS, alkyl sulphate, alcohol ethoxy sulphate and the like.

In particular, it is contemplated that an improved tolerance towards a non-ionic surfactant alcohol ethoxylate, a commercially available example of which is Dobanol®, may be indicative of improved wash performance.

any detergent composition in combination with one of the above detectors of enzymatic activity.

It will be understood that the screening criteria used in the filter assay of the invention may be chosen so as to comply with the desired properties or uses of the enzymes to be screened. For instance, in a screening for lipases of particular use in the paper and pulp industry, it may be relevant to screen for an acid lipase having an increased temperature stability. This may be performed by using a buffer with acidic pH (e.g. pH 4) and/or incubate under higher temperature before or under the assay.

The host cells produced in step (c) may be subjected to further rounds of mutagenesis as defined in steps (a)-(c) above, conveniently by using more stringent selection criteria than employed in a previous mutagenesis treatment.

The host cells selected for in step (c) may be used directly for the production of the variant of the lipolytic enzyme. Alternatively, DNA encoding the variant may be isolated from the host cell and inserted into another suitable host cell, conveniently by use of the procedure described below in the section entitled "Expression of a variant of the invention", in which suitable host cells are also listed.

Localized random mutagenesis

In accordance with the invention the random mutagenesis may advantageously be located to a part of the parent lipolytic enzyme in question. This may, e.g., be advantageous when a certain region of the enzyme has been identified to be of particular importance for a given property of the enzyme, and which, when modified, is expected to result in a variant having improved properties. Such region may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

during hydrolysis where the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface.

The lipid contact zone contains a binding area for the lipid substrate which is the part of the lipid contact zone to which the single lipid substrate molecule binds before hydrolysis. This binding area again contains an acyl-binding hydrophobic cleft and a so-called hydrolysis pocket, which is situated around the active site Ser, and in which the hydrolysis of the lipid substrate is believed to take place. In all lipases known today the lipid contact zone is easily recognized, e.g. from a three-dimensional structure of the lipase created by suitable computer programs. The conformation of an inactive and activated lipase, respectively, is shown in Figs. 1 and 2 of WO 92/05249.

The lipid contact zone of the *Humicola lanuginosa* lipase discussed in detail in the present application is defined by amino acid residues 21-25, 36-38, 56-62, 81-98, 110-116, 144-147, 172-174, 199-213 and 248-269. These residues have been identified on the basis of computer model simulations of the interaction between the lipase and a lipid substrate.

The lipid contact zone of other lipolytic enzymes is defined by

a) calculating the hydrophobic vector of the 3-D molecular structure,

b) making a cut perpendicular to the vector through the Cα-atom of the second amino acid residue after the active site serine in the linear sequence, and

c) including all residues with at least one atom on that side of the cut to which the vector points, and

d) selecting from those residues, those which have at least one atom within 5 Ångström of the surface of the protein (in case of a lipase in either its open or closed form).

question, but also an enzyme encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Furthermore, the term is intended to indicate an enzyme which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the enzyme in question.

Of particular interest as a parent lipolytic enzyme is a lipase derivable from a strain of *H. lanuginosa*, e.g. the *H. lanuginosa* strain DSM 4109, or an analogue of said lipase, a strain of *Rh. mucor*, or a strain of *C. antarctica*.

In the present context the term "analogue" is intended to include a polypeptide which comprises an amino acid sequence differing from that of the *H. lanuginosa* lipase by one or more amino acid residues, and which is at least 70% homologous with the amino acid sequence of said lipase, (determined as the degree of identity between the two sequences), such as at least 75%, 80%, 90% or 95% homologous, is immunologically cross reactive with said lipase, and/or which is encoded by a DNA sequence hybridizing with an oligo nucleotide probe prepared on the basis of the amino acid sequence of said lipase or of a DNA sequence encoding said lipase.

The analogue may be a derivative of the *H. lanuginosa* lipase, e.g. prepared by modifying a DNA sequence encoding the lipase resulting in the addition of one or more amino acid residues to either or both the N- and C-terminal end of the lipase, substitution of one or more amino acid residues at one or more different sites in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the lipase or at one or more sites in the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the amino acid sequence. The modification of the DNA sequence may be performed by site-directed or by random mutagenesis or a combination of these techniques in accordance with well-known procedures.

The localized random mutagenesis may be performed in one or more of these regions, and is preferably performed in at least two of the regions.

The parent lipolytic enzyme to be modified in accordance with the present invention may be derivable from a bacterium. For instance, the DNA sequence encoding the parent lipolytic enzyme may be derivable from a strain of *Pseudomonas* spp., such as *P. cepacia*, *P. alcaligenes*, *P. pseudoalcaligens*, *P. mendocina* (also termed *P. putida*), *P. syringae*, *P. aeroginosa* or *P. fragi*, a strain of *Bacillus* spp., e.g. *B. subtilis* or *B. pumilus* or a strain of *Streptomyces* sp., e.g. *S. scabies*.

The parent bacterial lipolytic enzyme may be a lipase derived from any of the above-mentioned species, e.g. a *Pseudomonas* lipase as described in EP 218 272, EP 331 376 and EP 407 225, or a cutinase, e.g. as described in WO 88/09367.

Variants of the invention

For ease of reference specific variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of aspartic acid for valine in position 96 is shown as:

Asp 96 Val or D96V

a deletion of aspartic acid in the same position is shown as:

Asp 96 * or D96*

and insertion of an additional amino acid residue such as lysine is shown as:

Asp 96 ValLys or D96VK

Multiple mutations are separated by pluses, i.e.:

Asp 96 Val + Glu 87 Lys or D96V+E87K

representing mutations in positions 96 and 87 substituting aspartic acid and glutamic acid for valine and lysine, respectively.

In addition the invention relates to a variant of the *H. lanuginosa* lipase obtainable from DSM 4109 or an analogue of said lipase, wherein the amino acid residue L264 has been replaced by an amino acid different from Leucine, i.e. any one of R, N, A, C, Q, E, G, H, I, K, M, F, P, S, T, W, Y, V, D.

Preferably, the variant according to the invention comprises at least one of the following mutations K46R, E57G, G61S, S83T, S58F, D62C, T64R, I90F, G91A, N92H, N94I, N94K, L97M, K98I, I100V, D102K, A121V, E129K, D167G, R205K, E210W, K237M, N259W, I252L, D254W, P256T, G263A, L264Q or T267W.

These positions have been found or is contemplated to be important for enzymatic activity and/or detergent tolerance. The numbering of the amino acid residues refers to the amino acid sequence of the mature lipase.

Preferably, the variant according to this aspect of the invention comprises at least one of the following mutations S83T, N94K, A121V, D167G, R205K.

It will be understood that the present invention encompasses variants of the parent *H. lanuginosa* lipase comprising a combination of two or more of the mutations defined herein, or a combination of one or more of the mutations defined herein with any of the mutations disclosed in WO 92/05249, WO 94/25577 and WO 94/01541.

In a further aspect the present invention relates to a variant of the *H. lanuginosa* lipase obtainable from DSM 4109 or an analogue thereof comprising at least one of the following mutations:

N94K+D96A
S83T+N94K+D96N
E87K+D96V
E87K+G91A+D96A
N94K+F95L+D96H

constructing these variants would be based on site-directed mutagenesis using suitable oligonucleotide probes. This method is exemplified in Examples 3-6.

Expression of a variant of the invention

According to the invention, a mutated DNA sequence encoding a variant lipolytic enzyme prepared by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding a variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), e.g. as described in WO 93/10249 the promoters of the *Bacillus*

medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, convenient accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding a variant of a parent lipolytic enzyme, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a variant of a parent lipolytic enzyme of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described below in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The trans-

Due to the decreased dependance to calcium and/or improved tolerance towards detergents or detergent components of the variant of the invention, the variant is particularly well suited for implementation into detergent compositions, e.g. detergent compositions intended for performance in the range of pH 7-13, particularly the range of pH 8-11.

Detergent Compositions

According to the invention, a lipase variant of the invention may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

tetraacetylethylenediamine (TAED) or nonanoyloxybenzene-sulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

(2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

(4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaAlSiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

(6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaAlSiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

(8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

(10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%
Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluensulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

(12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaA1SiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

(15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

(16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

(17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

(18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

(19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g.

polycarboxylates, polyacetyl carboxylates and polyhydroxsulphonates.

Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers and their salts.

The dishwashing detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite and hypobromite as well as chlorinated trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

The oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED and glycerol triacetate.

The dishwashing detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), e.g. a polyol such as e.g.propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester.

The dishwashing detergent composition may also comprise other enzymes, in particular an amylase, a protease and/or a cellulase.

The invention is further described in the following examples which are not, in any way, intended to limit the scope of the invention as claimed.

MATERIALS AND METHODS

Humicola lanuginosa DSM 4109 available from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-3300 Braunschweig, Federal Republic of Germany.

pYESHL is a yeast/*E. coli* shuttle vector that expresses and secretes a low level of the *H. lanuginosa* lipase in yeast. More specifically pYESHL is a derivative of pYES2 (purchased from Invitrogen Corp., UK) in which the GAL1 promoter was excised and the Humicola lanuginosa lipase gene and the TPI (triose phosphate isomerase) promoter from S. cerevisiae (Alber, T. and Kawasaki, G., J.Mol.Appl. Genet 1, 419-434 (1982) were cloned between the SphI and XbaI sites. A restriction map of pYESHL is shown in Fig. 1.

Low calcium filter assay

Procedure

1) Provide SC Ura$^-$ replica plates (useful for selecting strains carrying the expression vector) with a first protein binding filter (Nylon membrane) and a second low protein binding filter (Cellulose acetate) on the top.

2) Spread yeast cells containing a parent lipase gene or a mutated lipase gene on the double filter and incubate for 2 or 3 days at 30°C.

3) Keep the colonies on the top filter by transferring the topfilter to a new plate.

4) Remove the protein binding filter to an empty petri dish.

b) Performing localized random mutagenesis

A mutagenic primer (oligonucleotide) is synthesized which corresponds to the part of the DNA sequence to be mutagenized except for the nucleotide(s) corresponding to amino acid codon(s) to be mutagenized.

Subsequently, the resulting mutagenic primer is used in a PCR reaction with a suitable opposite primer. The resulting PCR fragment is purified and digested and cloned into the shuttle vector. Alternatively and if necessary, the resulting PCR fragment is used in a second PCR reaction as a primer with a second suitable opposite primer so as to allow digestion and cloning of the mutagenized region into the shuttle vector. The PCR reactions are performed under normal conditions.

DNA sequencing was performed by using applied Biosystems ABI DNA sequence model 373A according to the protocol in the ABI Dye Terminator Cycle Sequencing kit.

EXAMPLES

EXAMPLE 1

Construction of random lipase variants

Random mutagenized libraries of the entire *H. lanuginosa* lipase gene and of amino acids (aa) 91-97 and 206-211 thereof were prepared as described in Materials and Methods above.

The amino acid regions 91-97 and 206-211 were chosen for the first round of localized mutagenesis since these regions have been found to be important for wash performance. Region 91-97 is a part of the lid region of the lipase and region 206-211 constitutes part of the hydrophobic cleft of the lipase.

One oligonucleotide was synthesized for each of these regions comprising 93% of the wild type nucleotides and 2.33% of each of the other three nucleotides at amino acid codons wanted to

low calcium positives from mutagenesis of aa 91-97 were sequenced.

The three other mutations (in codon 83, 103, 145), outside the mutagenized region, can be explained by PCR misincoorperation, allthough the mutation of S83T is a transversion which is quite unusual for PCR misincoorperations.

Table 2

Strain number	Variant type						
59	I			G91A	N94K		D96A
60	II	S83T			N94K		D96N
61	II	S83T			N94K		D96N
62	III		E87K				D96V
63	IV		E87K	G91A			D96V
64	II	S83T			N94K		D96N
65	III		E87K				D96V
67	V				N94K	F95L	D96H
69	V				N94K	F95L	D96H
71	III		E87K				D96V
72	II	S83T			N94K		D96N

Table 2: Strain number refers to the originally picked clones cloned into Aspergillus expression vector pAHL. Variant type refers to identical clones, which probably have arisen during amplification of the random mutagenized library. Variant types I and II are active in 0.01% Dobanol™25-7 while the rest are inactive like wild type.

EXAMPLE 2

Analogously to the method described in Example 1, the following variants were constructed by random mutagenesis. The actual screening criteria used for selecting some of the variants are also described.

D167G+E210V

5mM EGTA,0.01% Dobanol™25-7,0.006% LAS
E87K+G91A+L93I+N94K+D96A

5mM EGTA,0.02% Dobanol™25-7
N73D+S85T+E87K+G91A+N94K+D96A
S83T+E87K+W89G+G91A+N94K+D96V
E87K+G91A+D96R+I100V
S83T+E87K+Q249R
E87K+G91A

EXAMPLE 3

Expression of Humicola lanuginosa lipase in *Aspergillus oryzae*
Cloning of Humicola lanuginosa lipase is described in EP 305 216. It also describes expression and characterization of the lipase in Aspergillus oryzae. The expression plasmid used is named p960.

The expression plasmid used in this application is identical to p960, except for minor modifications just 3' to the lipase coding region. The modifications were made the following way: p960 was digested with NruI and BamHI restriction enzymes. Between these two sites the BamHI/NheI fragment from plasmid pBR322, in which the NheI fragment was filled in with Klenow polymerase, was cloned, thereby creating plasmid pAO1 (figure 2), which contains unique BamHI and NheI sites. Between these unique sites BamHI/XbaI fragments from p960 was cloned to give pAHL (figure 3).

PCR Handle (=D): 5'-GGTCATCCAGTCACTGAGAC-3'

Helper 1 and helper 2 are complementary to sequences outside the coding region, and can thus be used in combination with any mutagenisation primer in the construction of a variant sequence.

All 3 steps are carried out in the following buffer containing: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 2.5 units Taq polymerase.

In step 1, 100 pmol primer A, 100 pmol primer B and 1 fmol linearized plasmid is added to a total of 100 µl reaction mixture and 15 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C are carried out.

The concentration of the PCR product is estimated on an agarose gel. Then, step 2 is carried out. 0.6 pmol step 1 product and 1 fmol linearized plasmid is contained in a total of 100 µl of the previously mentioned buffer and 1 cycle consisting of 5 minutes at 95°C, 2 minutes at 37°C and 10 minutes at 72°C is carried out.

To the step 2 reaction mixture, 100 pmol primer C and 100 pmol primer D is added (1 µl of each) and 20 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C are carried out. This manipulation comprised step 3 in the mutagenisation procedure.

Isolation of mutated restriction fragment

The product from step 3 is isolated from an agarose gel and re-dissolved in 20 µl H_2O. Then, it is digested with the restriction enzymes BamHI and BstXI in a total volume of 50 µl with the following composition: 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 10 units of BamHI and 10 units of BstXI. Incubation is at 37°C for 2 hours. The 733 bp BamHI/BstXI fragment is isolated from an agarose gel.

pAHLA121V 5'-CCTTAACGTATCAACTACAGACCTCCA-3'

pAHLR205K/E210Q 5'-GCTGTAACCGAATTGGCGCGGCGGGAGCTTAGGG-ACAATATC-3'

pAHLN73D/S85T/E87K/G91A/N94K/D96A

5'-TATTTCTTTCAAAGCGAACTTAAGATTAGCGATC-CAGTTCTTTATAGTACGAGAGCCACGGAA-AGAGAGGACGATCAATTTGTCCGTGTTGTCGAG-3'

pAHLS83T/E87K/W89G/G91A/N94K/D96V

5'-TATTTCTTTCAAAACGAACTTAAGATTAGCGATA-CCGTTCTTTATGGAACGAGTGCCACGGAAAGA-3'

pAHLE87K/G91A/D96R/I100V

5'-GCAAATGTCATTAACTTCTTTCAATCTGAAGTTAA-GATTAGCGATCCAGTTCTTTATGGAACGAGA-3'

pAHLS83T/E87K 5'-CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-AAAGA-3'

pAHLE87K/G91A 5'-GAAGTTAAGATTAGCGATCCAGTTCTTTATGGAA-CGAGA-3'

pAHLS83T/E87K 5'-CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-AAAGA-3'

pAHLQ249R 5'-CGGAATGTTAGGTCTGTTATTGCCGCC-3'

EXAMPLE 5

Construction of plasmids expressing combination analogues of *Humicola lipase*

The plasmids pAHLD167G/E210V
pAHLA121V/R205K/E210Q
and pAHLS83T/E87K/Q249R

are constructed by performing two successive mutagenisation steps using the appropriate primers.

EXAMPLE 6

Expression of lipase analogues in *Aspergillus*

Transformation of *Aspergillus oryzae* (general procedure)

7 days at 37°C spores are picked, suspended in sterile water and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation are stored as a defined transformant.

Expression of lipase analogues in *A. oryzae*

The plasmids described above are transformed into A. oryzae IFO 4177 by cotransformation with p3SR2 containing the amdS gene from A. nidulans as described in the above example. Protoplasts prepared as described are incubated with a mixture of equal amounts of expression plasmid and p3SR2, approximately 5 μg of each are used. Transformants which could use acetamide as sole nitrogen source are reisolated twice. After growth on YPD for three days, culture supernatants are analyzed using an assay for lipase activity. The best transformant is selected for further studies and grown in a 1 l shake-flask on 200 ml FG4 medium (3% soy meal, 3% maltodextrin, 1% peptone, pH adjusted to 7.0 with 4 M NaOH) for 4 days at 30°C.

EXAMPLE 7

Purification of lipase variants of the invention

Assay for lipase activity :

A substrate for lipase was prepared by emulsifying glycerine tributyrat (MERCK) using gum-arabic as emulsifier.

Lipase activity was assayed at pH 7 using pH stat method. One unit of lipase activity (LU/mg) was defined as the amount needed to liberate one micromole fatty acid per minute.

Step 1:- Centrifuge the fermentation supernatant, discard the precipitate. Adjust the pH of the supernatant to 7 and add gradually an equal volume of cold 96 % ethanol. Allow the mixture to stand for 30 minutes in an ice bath. Centrifuge and discard the precipitate.

Method:	3 cycles with overnight drying between each cycle	
Wash liquor:	100 ml per beaker	
Swatches:	6 swatches (3.5 x 3.5 cm) per beaker	
Fabric:	100% cotton, Test Fabrics style #400	
Stain:	Lard coloured with Sudan red (0.75 mg dye/g of lard). 6 µl of lard heated to 70°C was applied to the centre of each swatch. After application of the stain, the swatches were heated in an oven at 75°C for 30 minutes. The swatches were then stored overnight at room temperature prior to the first wash.	
Detergent:	LAS (Nansa 1169/P, 30% a.m.)	1.17 g/l
	AEO (Dobanol™25-7)	0.15 g/l
	Sodium triphosphate	1.25 g/l
	Sodium sulphate	1.00 g/l
	Sodium carbonate	0.45 g/l
	Sodium silicate	0.15 g/l
pH:	10.2	
Lipase conc.:	0.075, 0.188, 0.375, 0.75 and 2.5 mg of lipase protein per litre	
Time:	20 minutes	
Temperature:	30°C	
Rinse:	15 minutes in running tap water	
Drying:	overnight at room temperature (~20°C, 30-50% RH)	
Evaluation:	after the 3rd wash, the reflectance at 460 nm was measured.	

Results

Dose-response curves were compared for the lipase variants and the native H. lanuginosa lipase. The dose-response curves were calculated by fitting the measured data to the following equation:

$$\Delta R = \Delta R_{max} \frac{C^{0.5}}{K + C^{0.5}} \qquad (I)$$

where ΔR is the effect expressed in reflectance units

Table 1

Variant	Improvement factor
E87K+D96V	1.2
S83T+N94K+D96N	2.3
N94K+D96A	2.7
E87K+G91A+D96A	2.6
N94K+F95L+D96H	3.3
D167G+E210V	5.0
E87K+G91A+L93I+N94K+D96A	1.3
E87K+G91A+D96R+I100V	5.2
E87K+G91A	5.0
N73D+E87K+G91A+N94I+D96G	1.3
S83T+E87K+G91A+N92H+N94K+D96M	3.8
K46R+E56R+G61S	1.9
D102K	0.2
D167G	1
N73D+E87K+G91A+N94I+D96G	1.3
E210R	2.7
E210K	5.5
E210W	1
N251W+D254W+T267W	0.8
S83T+E87K+G91A+N92H+N94K+D96M	3.8
E56R+I90F+D96L+E99K	4.8
D57G+N94K+D96L+L97M	1.9

} ~ random

} ~ random

Fowler et al., Molec. gen. Genet., 133, pp. 179-191, 1974.

Brady et al., "A Serine Protease Triad Forms the Catalytic Centre of a Triacylglycerol Lipase", Nature 343, 1990, pp. 767-770, 1990.

Tilbeyrgh, H. van, Egloff, M.-P., Martinez,C., Rugani,N., Verger,R. and Cambillau (1993) Nature 362, p. 814-820. Interfacial activation of the lipase-prolipase complex by mixed micelles revealed by X-ray crystallography.

Hudson et al., Practical Immunology, Third edition, Blackwell Scientific Publications, 1989

Alber, T. and Kawasaki, G., J.Mol.Appl. Genet 1, 419-434 (1982)

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ATG AGG AGC TCC CTT GTG CTG TTC TTT GTC TCT GCG TGG ACG GCC TTG   48
Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
     -20                 -15                 -10

GCC AGT CCT ATT CGT CGA GAG GTC TCG CAG GAT CTG TTT AAC CAG TTC   96
Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
  -5                  1                   5                  10

AAT CTC TTT GCA CAG TAT TCT GCA GCC GCA TAC TGC GGA AAA AAC AAT  144
Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
                15                  20                  25

GAT GCC CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC  192
Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
            30                  35                  40

GAG GTA GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT  240
Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
        45                  50                  55

GGA GTG GGC GAT GTC ACC GGC TTC CTT GCT CTC GAC AAC ACG AAC AAA  288
Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
    60                  65                  70

TTG ATC GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAG AAC TGG ATC  336
Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
75                  80                  85                  90

GGG AAT CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC  384
Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
                95                 100                 105

TGC AGG GGA CAT GAC GGC TTC ACT TCG TCC TGG AGG TCT GTA GCC GAT  432
Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
            110                 115                 120
ACG TTA AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT  480
Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
        125                 130                 135

CGC GTG GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT  528
Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
    140                 145                 150

GCC GGA GCA GAC CTG CGT GGA AAT GGG TAT GAT ATC GAC GTG TTT TCA  576
Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
155                 160                 165                 170

TAT GGC GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC  624
Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
                175                 180                 185

GTA CAG ACC GGC GGA ACA CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT  672
Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
            190                 195                 200

GTC CCT AGA CTC CCG CCG CGC GAA TTC GGT TAC AGC CAT TCT AGC CCA  720
Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
        205                 210                 215

GAG TAC TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAT  768
Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
    220                 225                 230

ATC GTG AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT  816
Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235                 240                 245                 250

AAC ATT CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGG  864
```

Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235 240 245 250

Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
255 260 265

Thr Cys Leu

6. The method according to claim 5, in which the host cell is a cell of a fungal or a bacterial strain.

7. The method according to claim 6, in which the host cell is a cell of the genus *Aspergillus*, such as *A. niger*, *A. oryzae* and *A. nidulans*, or a cell of the genus *Saccharomyces*, e.g. *S. cereviciae*.

8. The method according to claim 6, in which the host cell is a cell of a gram-positive bacterial strain, e.g. of the genus *Bacilllus*, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus*, or a cell of a gram-negative bacterial strain, such as *E.coli*.

9. The method according to claim 1, in which the mutated lipolytic enzyme has an improved tolerance towards a non-ionic, anionic, kationic, zwitterionic or amphoteric surfactant.

10. The method according to claim 9, in which the non-ionic surfactant is an alcohol ethoxylate and/or the anionic surfactant is LAS or an alkyl sulphate.

11. The method according to claim 1, wherein host cells screened in step (c) are subjected to a second mutagenesis treatment, to rescreening, to reisolation and/or to recloning.

12. The method according to any of claims 1-11, in which the random mutagenesis is localized to a part of the DNA sequence encoding the parent lipolytic enzyme.

13. The method according to any of claims 1-12, in which the parent lipolytic enzyme is a lipase, an esterase, a cutinase or a phospholipase.

22. The method according to claim 16, wherein the parent lipolytic enzyme is derivable from a bacterium.

23. The method according to claim 22, wherein the DNA sequence encoding the parent lipolytic enzyme is derivable from a strain of *Pseudomonas* spp., such as *P. cepacia*, *P. alcaligenes*, *P. pseudoalcaligens* or *P. fragi*. or from a strain of *Bacillus*.

24. A variant of a lipolytic enzyme prepared by the method according to any of claims 1-23.

25. A variant according to claim 24 which is a variant of the *H. lanuginosa* lipase obtainable from DSM 4109 or a analogue thereof, which comprises a mutation in at least one of the following positions:

K46, E56, S58, G61, T64, N73, S83, I90, G91, N92, N94, D96, L97, K98, E99, I100, D102, A121, E129, D167, R205, E210, K237, N251, I252, D254, P256, G263, L264 or T267.

26. A variant of the *H. lanuginosa* lipase obtainable from strain DSM 4109 or a analogue of said lipase, which carries a mutation in at least one of the regions defined by the amino acid residues 56-64, 83-100 or 205-211.

27. A variant according to claim 26, which comprises at least one of the following mutations:

K46R, D57G, S58F, G61S, D62C, T64R, S83T, I90F, G91A, N92H, N94I, N94K, L97M, K98I, I100V, D102K, A121V, E129K, D167G, R205K, E210W, K237M, N259W, I252L, D254W, P256T, G263A, L264Q or T267W.

28. A variant of the *H. lanuginosa* lipase obtainable from DSM 4109 or an analogue thereof comprising at least one of the following mutations:

N94K+D96A
S83T+N94K+D96N

30. A DNA construct encoding a *H. lanuginosa* lipase variant according to any of claims 24-28.

31. A vector harbouring a DNA construct according to claim 29 or 30.

32. The vector according to claim 31, which is a plasmid or a bacteriophage.

33. The vector according to claim 31 or 32, which is an expression vector further comprising DNA sequences permitting expression of the variant of the parent lipolytic enzyme.

34. A host cell harbouring a DNA construct according to claim 29 or 30 or a vector according to any of claims 31-33.

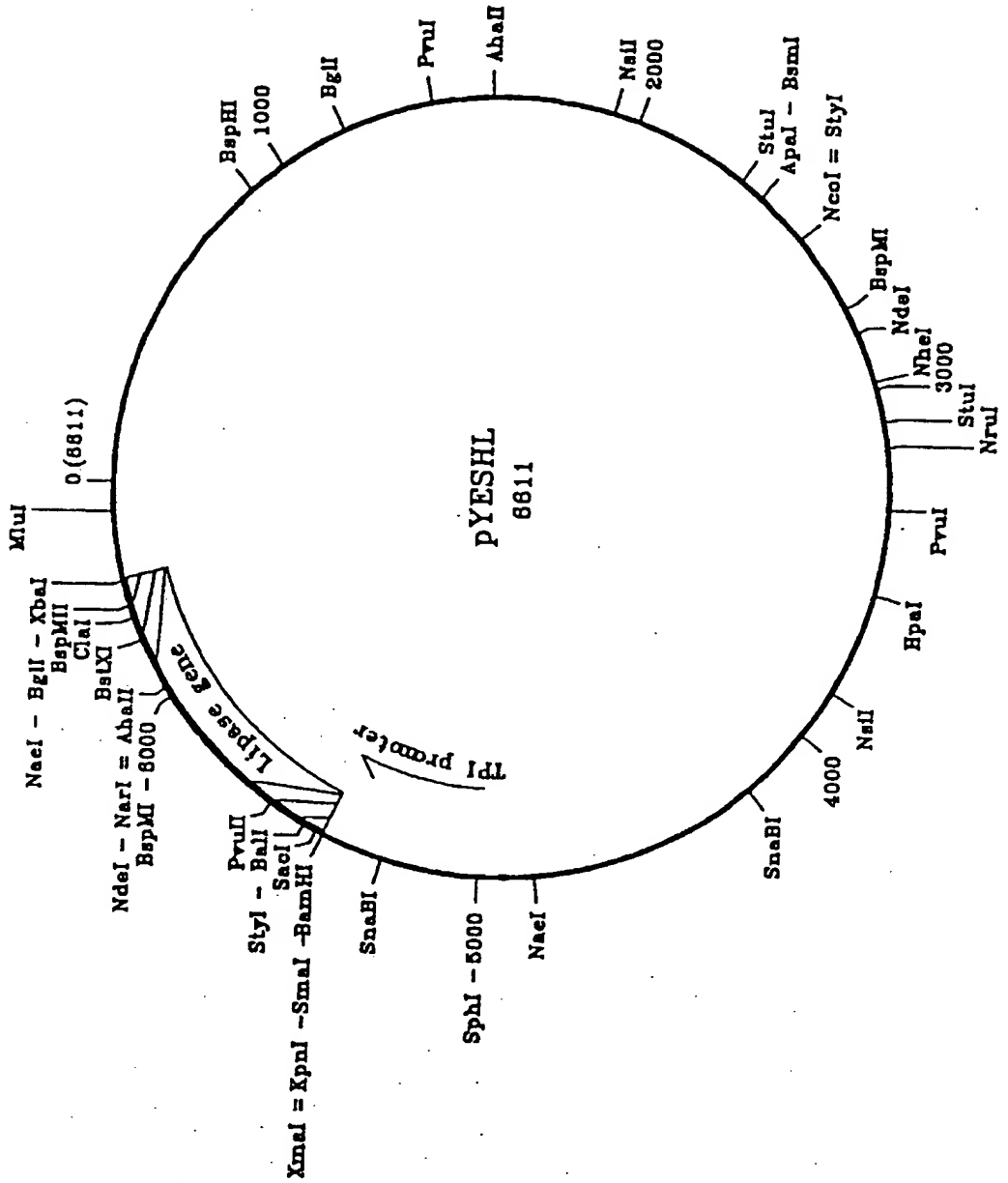
35. The cell according to claim 34, which is a microbial cell.

36. The cell according to claim 35, which is a cell of a fungal or a bacterial strain.

37. The cell according to claim 36, which is a cell of the genus *Aspergillus*, such as *A. niger*, *A. oryzae* and *A. nidulans*, or a cell of the genus *Saccharomyces*, e.g. *S. cereviciae*.

38. The cell according to claim 36, which is a cell of a gram-positive bacterial strain, e.g. of the genus *Bacilllus*, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus*, or a cell of a gram-negative bacterial strain, such as *E.coli*.

39. A method of producing a variant of a parent lipolytic enzyme which has a decreased dependance to calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme, which method comprises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4000
5000
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TPI promoter
Lipase gene
MluI
NaeI – BglII – XbaI
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ClaI
BsiXI
AbaII
NdeI – NarI = AbaII
BspMI – 8000
PvuII
StyI – BalI
SacI
XmaI = KpnI –SmaI –BamHI
SnaBI
SphI – 5000
NaeI
SnaBI
NaII
EpaI
PvuI
NruI
NheI
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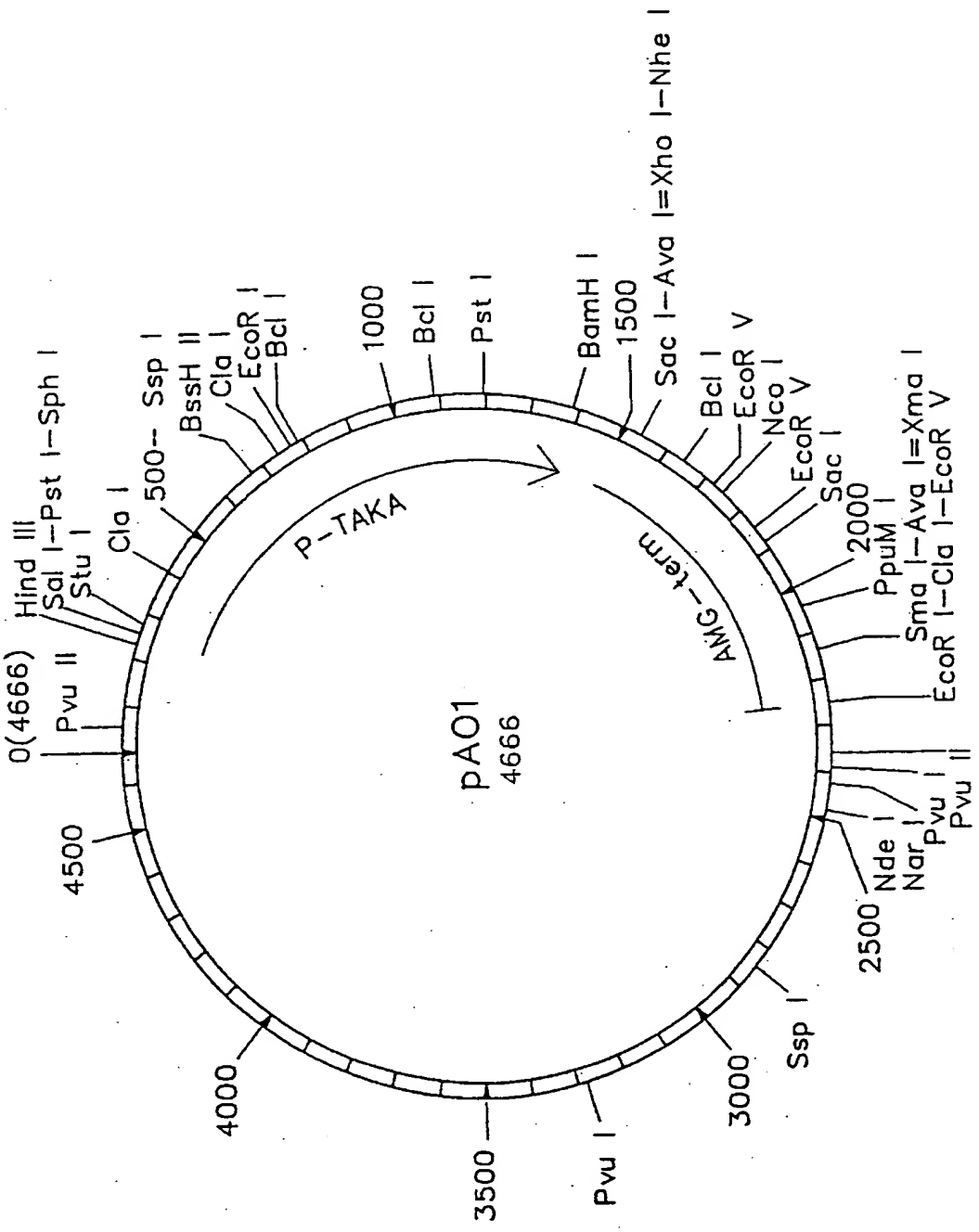
BspHI

Fig. 1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Sal I-Pst I-Sph I
Stu I
Pvu II
Cla I
BssH II
Ssp I
EcoR I
Cla I
Bcl I
Bcl I
Pst I
BamH I
Sac I-Ava I=Xho I-Nhe I
Bcl I
EcoR V
Nco I
EcoR V
Sac I
PpuM I
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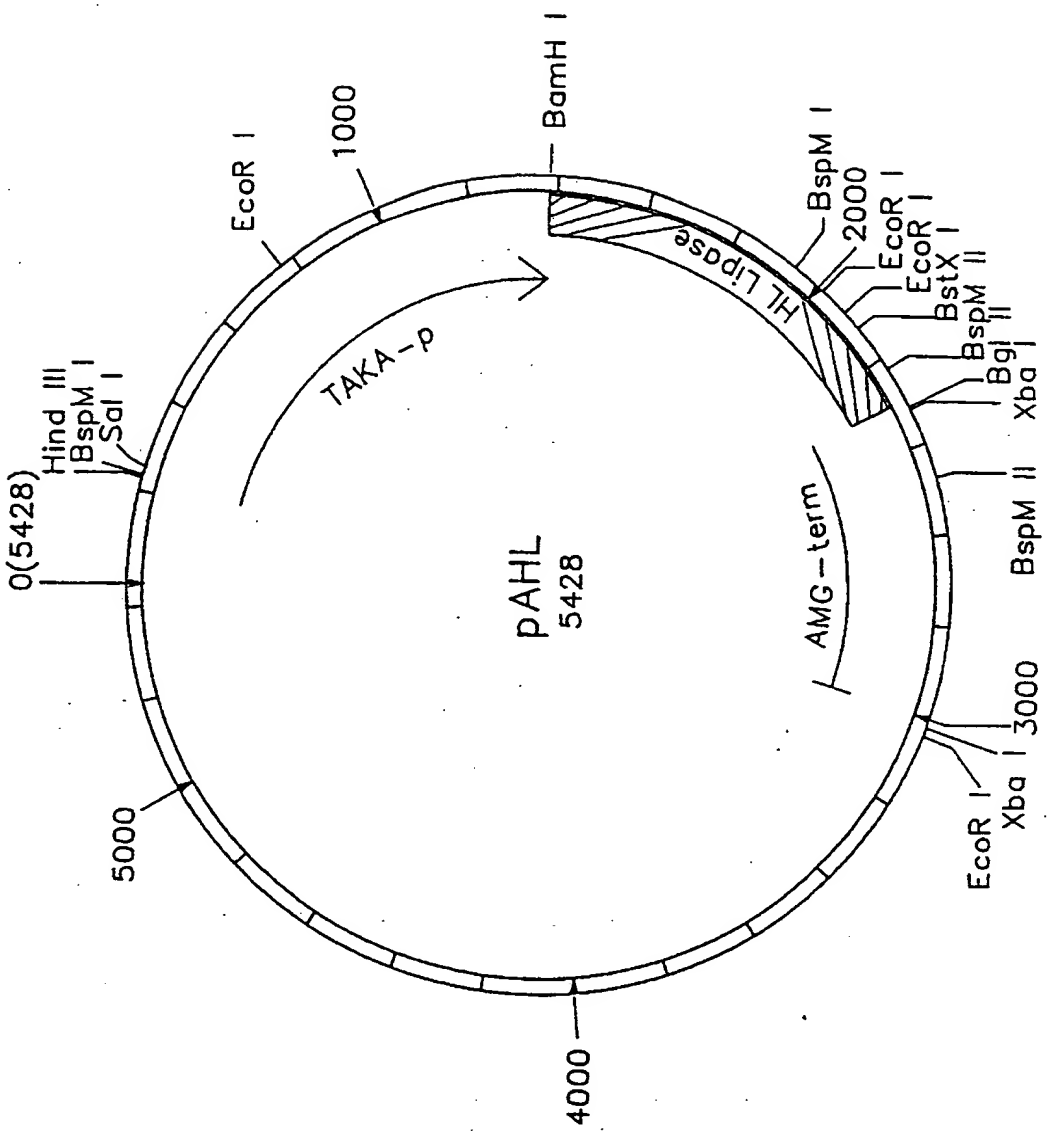
Ssp I
Pvu I

Fig. 2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BspM II
Bgl I
Xba I
HL Lipase
TAKA-p
Hind III
BspM I
Sal I
0(5428)
pAHL
5428
AMG-term
BspM II
3000
EcoR I
Xba I
5000
4000

Fig. 3

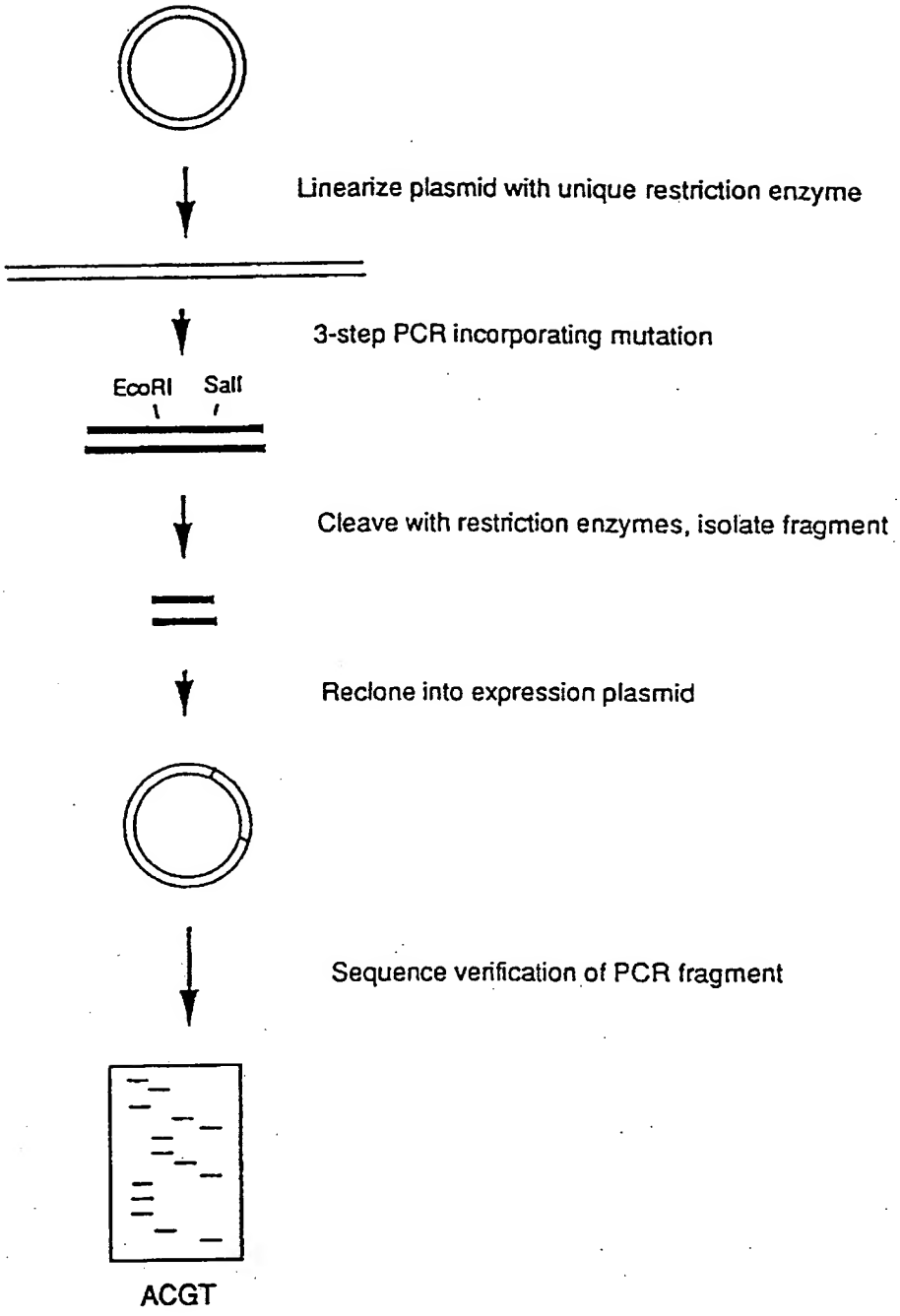
Linearize plasmid with unique restriction enzyme
3-step PCR incorporating mutation
EcoRI
SalI
Cleave with restriction enzymes, isolate fragment
Reclone into expression plasmid
Sequence verification of PCR fragment
ACGT

Fig. 4

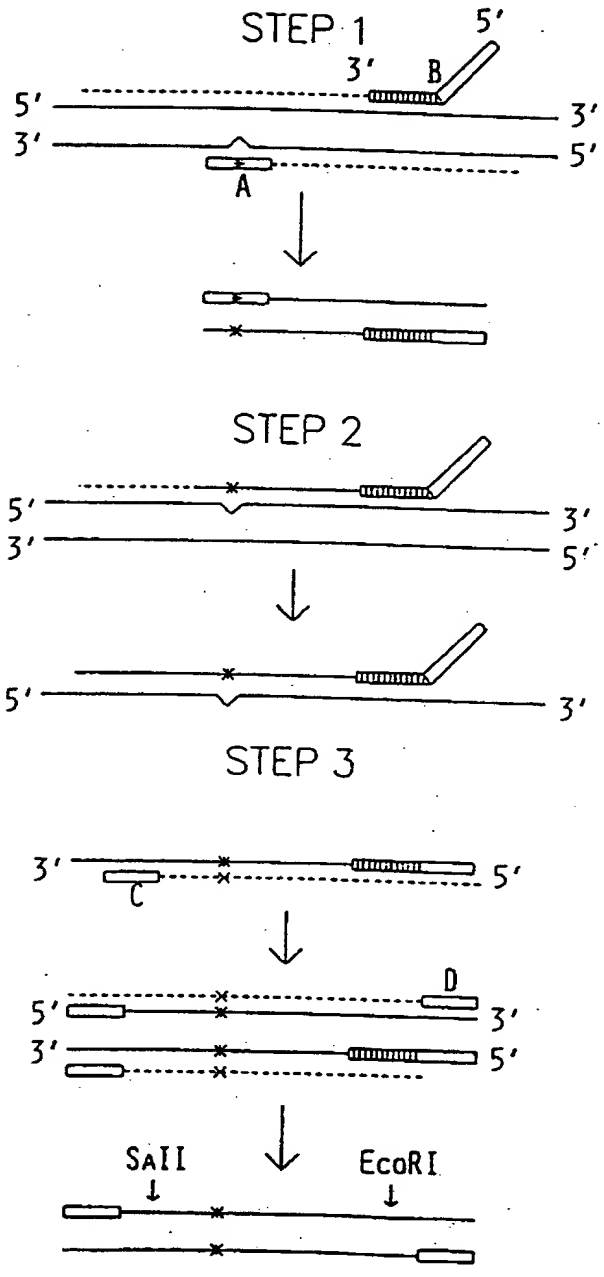

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00079

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/55, C12N 9/20, C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WPIL, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0525610 (SOLVAY ENZYMES GMBH & CO. KG), 3 February 1993 (03.02.93), the claims --	1-46
X	WO, A1, 9205249 (NOVO NORDISK A/S), 2 April 1992 (02.04.92), page 2, line 22 - page 3, line 14; page 18, line 23 - line 32, the claims --	1-46
P,X	WO, A1, 9414964 (UNILEVER N.V.), 7 July 1994 (07.07.94), page 9, line 4 - line 11, the claims --	1-46

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 June 1995	19 -06- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

Information on patent family members

03/05/95

International application No.
PCT/DK 95/00079

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0525610	03/02/93	DE-A- 4224125	28/01/93
WO-A1- 9205249	02/04/92	AU-B- 657278	09/03/95
		AU-A- 8617291	15/04/92
		CA-A- 2092615	14/03/92
		EP-A- 0548228	30/06/93
		JP-T- 6501153	10/02/94
WO-A1- 9414964	07/07/94	NONE	

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